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c0009 ***Production of Dicarboxylic Acid Platform Chemicals Using Yeasts: Focus on Succinic Acid***

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s0010 ***9.1 Markets and Applications***

s0015 ***9.1.1 Drivers for Biobased Chemicals***

p0010 The continuously expanding human population and the rising average income of the population cause an increasing demand on energy and raw materials. The steady depletion in fossil feedstocks, the impact of environmental and geopolitical disturbances on price and security of their supply, ecological problems due to emissions of CO₂ and other greenhouse gases, and the consequent impact on climate change are forcing advanced industrialized economies to search and develop alternative and renewable sources for energy, transport fuels, and chemicals. Over the last decade, governments, international organizations, and industry associations [AU1] have issued strategic vision documents and initiated R&D programs for a major transition from the current economy that relies on fossil resources such as crude oil and natural gas to a so-called bioeconomy, based on renewable biomass from agriculture, forestry, and agro-industrial waste streams. Biological raw material from plants and trees, or waste, is renewable in the short term (less than 10 years), as opposed to fossil material renewable in 10 million years. Biobased products can thus make a sizable contribution to reduction in CO₂ emissions and in the dependence on fossil resources, such as crude oil, natural gas, and coal. Moreover, industrial biotechnology frequently shows significant performance benefits compared with conventional chemical technology, such as a higher reaction rate, increased conversion efficiency, improved product purity, lowered energy consumption, and significant decrease in

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chemical waste generation. So far, the use of biomass for production of transportation biofuels has attracted most political and public attention, although industrial biotechnology allows for the coproduction of a wide range of biobased chemicals from renewable biomass rather than fossil resources, without requiring the massive amounts of raw materials of the fuels market.

p0015 The diversity of biobased products that can be produced covers a broad range of intermediate products, product components, and ready-made products, eg, biobased plastics, biolubricants, biofibers for textiles, composite materials for construction and automotive, chemical and pharmaceutical building blocks, organic acids, amino acids, and enzymes. In addition, biobased products may offer specific innovative properties that have advantages over other products. For example, in sensitive environments, biodegradable lubricants that are nontoxic to soil and water can be used. The addressable market is thus extremely large. It has been estimated by the OECD that the economic contribution of biotechnology in 2030 will be greatest in industrial applications, with 39% of the total potential gross value added from biotechnology, followed by primary production with 36% of the total and health applications at 25% of the total (OECD, 2009). This estimate conflicts sharply with an OECD estimate of the distribution of R&D expenditures by businesses in 2003, where of the vast majority of private sector R&D investment, 87%, went to health applications, with only 2% of biotechnology R&D expenditures spent on industrial applications (OECD, 2009). Since then, the R&D efforts and the development of new technology have experienced an unprecedented dynamism fueled not only by the need to meet the goals, but also by benefiting from the advances in the biological sciences, including a wealth of advanced genomic and molecular tools that are now available at reasonable cost as a result of the investments already made in the advancement of pharmaceutical biotechnology and biomedicine. This chapter illustrates the multidisciplinary endeavor required to develop sustainable processes that respect the environment, improve quality of life, and are competitive in the marketplace. The ultimate goal is the development of a clean (bio)chemical technology, starting from renewable raw materials and energy, with minimal waste generation, and maximal productivity and competitiveness. It encompasses the integrated application of disciplines such as biochemistry, bioinformatics, genetics, systems biology, and bioprocess engineering to develop useful processes and products, based on the catalytic action of microbial cells. The transformation of renewable carbon sources to a variety of biotechnological end products and precursors for chemical processes has been generally limited to products of natural metabolic pathways. Even when highly sophisticated biocatalysts have been developed, the genetic changes were targeted to enhancing the productivity and yields. The emerging field of synthetic biology is opening new opportunities by reassembling existing metabolic pathways and even introducing synthetic metabolic pathway modules into living systems.

p0020 Among the possible entry points of biobased products in the value chain from feedstocks to end products, platform chemicals seem to provide a particularly promising opportunity. These

biobased intermediate compounds can be then transformed in the same diversity of end products currently produced from oil-derived building blocks. Dicarboxylic acids are an example of such platform chemicals.

s0020 **9.1.2 Current Uses and Production of Dicarboxylic Acids**

s0025 *Succinic acid*

p0025 Succinic acid is a key building block for a wide range of secondary chemicals and finds use in chemical, pharmaceutical, food, and agricultural industries. Succinic acid is a C4-dicarboxylic acid, presently produced primarily from petroleum feedstock. Four carbon dicarboxylic acids have the potential to be key building blocks for deriving both commodity and specialty chemicals. The basic chemistry of succinic acid is similar to that of the petrochemically derived maleic acid/anhydride, which has a market of over 1.6 million metric tons and is used in the production of other chemicals such as succinic anhydride, 1,4-butanediol (BDO), fumaric acid, diethyl maleate, glyoxylic acid, and other plastics. Succinic acid has remained a largely underexploited market, despite its potential as a platform molecule, primarily due to the high production cost associated with the petroleum-derived process. Its average market price typically fluctuates between \$3–8 per kilo, significantly linked to the fluctuations of oil prices and more than double the typical price of maleic anhydride. The current market for succinic acid is around \$240 million (TMR, 2013). Biobased succinic acid is estimated to expand the succinic acid marketplace with emerging applications in the production of biobased 1,4-BDO, gamma-butyrolactone and tetrahydrofuran (THF) and their derivatives, and use as building block of polyesters through the polymerization of succinic acid with diols. Indeed, in 2012 the market of succinic acid was around 60 kton/year and was expected to grow to 665 kton/year until 2020. For example, succinic acid together with 1,4-BDO forms polybutylene succinate (PBS), which is biodegradable polyester with similar properties to polyethylene terephthalate, but could also substitute polyethylene, polylactic acid and polypropylene in some applications. The market for PBS is currently small, around 15 kton/year; it is expected to increase quickly as a 100% biobased product, using biobased succinic acid and biobased 1,4-BDO, which can also be derived from biobased succinic acid.

p0030 Many different microorganisms have been screened and studied for succinic acid production from various carbon sources. Among them, *Anaerobiospirillum succiniciproducens*, *Actinobacillus succinogenes*, *Mannheimia succiniciproducens*, and *Basfia succiniciproducens* have been most intensively studied due to their ability to produce a relatively large amount of succinic acid. Usually, these natural succinate-producing strains require complex and rich nutrient media, which increase substrate requirements and cost, and the productivity rarely reaches the desired target for feasible operation (Beauprez et al., 2010). As conventional strain improvement is often not sufficient, metabolic engineering is also applied to improve strain productivity, but this approach may be delayed by the lack of molecular genetic tools for these particular organisms (BREW, 2006). There has been much effort in developing

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recombinant *Escherichia coli* strains, which are capable of enhanced succinic acid production under aerobic and anaerobic conditions. In contrast to natural succinic acid producers, which employ only one major pathway for succinic acid production, *E. coli* uses a total of six biosynthetic routes. Pathway engineering has been performed in order to direct carbon flow predominantly to succinic acid, assisted by in silico metabolic pathway analysis of engineered strains. The microbe had been optimized to produce succinate salts from sugar and CO₂, with 25% of the carbon in the product coming from CO₂. Overall, the biosynthesis consumes CO₂ rather than generating it. This process has already been validated using wheat-derived glucose at 350,000 L (Ritter, 2011). In an ideal situation, the fermentation would be run at low pH, most preferably without requiring any neutralization to convert the resulting sodium succinate to the free acid required for subsequent derivatization. The conversion of the salt to the free acid adds significant costs, and undesired salts are produced. As such, low pH-tolerant yeast strains have been successfully engineered to produce succinic acid with less by-products (van den Tweel, 2010; Ritter, 2011). The use of yeast could also allow using retrofit bioethanol facilities for the production of succinic acid. The major fermentation feedstock is still glucose, but ongoing efforts are aiming at introducing cheaper, less-processed feedstocks including sorghum grits and hydrolyzed agricultural and forest residues, such as corn stover, bagasse, rice straw, and wood chips. Several companies have announced the launch of demonstration facilities (Table 9.1), which are supplying succinic acid to the market, particularly to strategic partners with which development of specific applications downstream the value chain is being performed.

s0030 *Fumaric acid*

p0035 Fumaric acid had an estimated annual production volume of 230 kton in 2013 (GVR, 2015) and is particularly used in food and animal feed for pH adjustment, preservation, and flavor enhancement. Fumaric acid derivatives have also been tested in a pharmaceutical application for treatment of psoriasis. Addition of fumaric acid to animal feed can lead to large reduction of the methane emissions by cattle (up to 70%). Fumaric acid contains a double bond and two carboxylic groups, which makes it very suitable for production of polyester and alkyd resins.

t0010 **Table 9.1: Demonstration-Scale Facilities for the Production of Biobased Succinic Acid.**

Company	Technology	Capacity (ton/year)
BioAmber	Genetically modified <i>E. coli</i> using glucose; currently developing an alternative yeast platform	30,000
Myriant	Genetically modified <i>E. coli</i> using glucose; planned future use of lignocellulosic sugars	10,000
Reverdia (joint venture between DSM and Roquette)	Genetically modified yeast using glucose; planned future use of lignocellulosic sugars	10,000
Succinity (joint venture between BASF and Corbion)	<i>Basfia succiniciproducens</i>	10,000

In the early 20th century, fumaric acid was biotechnologically produced at industrial scale, but after the Second World War, the production process was switched to a petrochemical route. Today, fumaric acid is produced in an amount exceeding 100kton/year by the chemical route from maleic anhydride. The cost of fumaric acid ranges between \$2.0–2.2/kg, which is [AU2] about 10% higher than the price of maleic anhydride. Fumaric acid is attractive for polymer production due to its nontoxic nature as well as its ability to give special properties as hardness to the polymer structure. The currently available native fumaric acid fungal producers represent a challenge for industrial fermentations due to filamentous morphology. The presently known recombinant hosts produce fumaric acid at low titer, rate, and yield. Fumaric acid is cited in the development pipeline of various companies currently focusing on succinic acid, including DSM and Myriant.

s0035 *Glutaric acid*

p0040 The applications of glutaric acid remain fairly limited for niche applications. It is produced as a by-product of the production process of adipic acid (about 2% of the output of an adipic acid plant is glutaric); however, it does not usually reach the market because it is converted into dibasic esters and sold as environmentally friendly solvents. The bulk price of glutaric acid ranges between \$10 and \$17/kg. Glutaric acid can be produced through various chemical routes, for example, from cyclopentane by oxidation with molecular oxygen and cobalt (III) catalysts or by ozonolysis; and from cyclopentanol–cyclopentanone by oxidation with oxygen and $\text{Co}(\text{CH}_3\text{CO}_2)_2$, with potassium peroxide in benzene, or with N_2O_4 or nitric acid. Together with succinic acid, glutaric acid is formed as a by-product during oxidation of cyclohexanol–cyclohexanone in the adipic acid production process. None of these routes yield a biobased product.

p0045 The company Rennovia (USA) has submitted a patent that discloses a method using chemical catalysis for the production of glutaric acid from carbohydrate-containing materials (WO2010144871). Briefly, pentoses, for example, those sourced from hemicellulose-containing raw materials, are oxidized into the corresponding pentaric acid in the presence of an oxidation catalyst. Thereafter, the pentaric acid is reacted with hydrogen in the presence of a hydrode-oxygenation catalyst and a halogen source (for example, hydrogen bromide or iodide) to yield glutaric acid. No biotechnological processes for the production of glutaric acid have been developed so far, despite ongoing early-stage research using *E. coli* or *Saccharomyces cerevisiae* as host. In the case of the *E. coli* platform, lysine and α -ketoglutarate have been used as co-substrates, with impact on the economic feasibility of the process.

s0040 *Itaconic acid*

p0050 Itaconic acid is an important building block in the chemical industry. It is a white crystalline powder and readily biodegrades in soil. Hence, it is an optimum substitute for petro-derived chemicals such as acrylic acid, maleic anhydride, or acetone cyanohydrin in various end-user

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industries. The demand for itaconic acid is high in the manufacturing of superabsorbent polymers, mainly used in diapers, adult incontinence, and feminine hygiene products. Itaconic acid is used as a cross-linking agent due to its ability to efficiently take part in addition polymerization. It also finds large application in seed coating, root dipping, ornamental gardens, food packaging, and artificial snow. Moreover, increasing demand for unsaturated polyester resins in pipes, artificial stones, electrical cabinets, and laminating resins is expected to increase the demand for itaconic acid. High price of itaconic acid is the major factor hampering the growth of itaconic acid market. Polyitaconic acid (a derivative of itaconic acid) has the potential to replace sodium tripolyphosphate in detergents. However, strong establishment of other phosphate-free builders impedes the growth of itaconic acid in detergents application. Other application segments include lubricant oil, adhesives, paints and coatings, pharmaceuticals, emulsifiers, herbicides, printing chemicals, and acrylic fiber. The global market is estimated to be worth around \$126 million/kg (TMR, 2015). The production in China has boomed, and as a result, the market price decreased to around \$2/kg or even lower (Boy and Lappe, 2012).

p0055 Itaconic acid is a biobased product mainly produced by fermentation using certain filamentous fungi (eg, *Ustilago*, *Helicobasidium*, and *Aspergillus*). A mixture of itaconic acid, citraconic acid, and citraconic anhydride is also obtained by reaction of succinic anhydride with formaldehyde at 200–500°C in the presence of alkali or alkaline earth hydroxides (could at least partially be biobased if biobased succinate is used as raw material for the production of succinic anhydride). Other methods involve carbonylation of propargyl chloride with metal carbonyl catalysts and thermal decomposition of citric acid, which is also a biobased chemical. *Aspergillus terreus* is the strain commonly used for the industrial production of itaconic acid. A significant amount of research has been put into the reduction of the production costs: the replacement of sugar, used as the carbon source, by cheaper alternative substrates such as cellulolytic biomass; optimizing the bioreactor type and configuration; deriving innovations by which the process becomes more energy saving; strain improvement by genetic and metabolic engineering, allowing the effective use of cheap alternative substrates, etc. Recent patent activity has particularly focused on the improvement of the producing strain, mainly by using recombinant DNA techniques, and several patents have been submitted worldwide in the last 10 years. There is a significant market opportunity for the development of biobased products from the C5 building block, itaconic acid. The major challenges are primarily associated with reducing the overall cost of the fermentation. It was estimated that in order to render the products derived from biobased itaconic acid competitive with petrochemical-derived products, the fermentation cost needed to be below \$1/kg, which is a significant technical challenge and should be undertaken with a longer-term perspective.

s0045 Other acids

p0060 The production of various other dicarboxylic acids using biotechnological routes has been being increasingly discussed. For example, although adipic acid is commercially the most important aliphatic dicarboxylic acid, no biobased product is yet on the market, with a current

- annual market of 2.5–3 million tons with an estimated value of \$5–7 billion. In large-scale production, the six carbon atoms of the adipic acid backbone are usually derived from benzene, which is hydrogenated to cyclohexane or phenol. Phenol can be hydrogenated to form cyclohexanol, while cyclohexane can be oxidized with air to a ketone–alcohol oil (KA oil, a mixture of cyclohexanol and cyclohexanone). Cyclohexanol, cyclohexanone, or KA oil is then oxidized with nitric acid to form adipic acid. Several processes have been suggested for the production of biobased adipic acid, and many of these proposed by advanced biotechnology companies, including Verdezyne, which achieved proof of concept production and recovery of adipic acid by an engineered *S. cerevisiae* from an alkane feedstock. Verdezyne DSM Venturing, [AU3] a corporate venture fund from DSM, has invested in Verdezyne, and DSM has recently announced it will invest in biobased adipic acid in the next years, targeting a 100–150 kton/year commercial scale target. Also Genomatica, a synthetic biology company that has developed intellectual targeting processes to produce over 20 intermediate and basic chemicals, including [AU4] adipic acid. Building on the success of the development of a feasible biobased succinic acid process, BioAmber and Myriant have also included biobased adipic acid in their pipeline. Other companies active in the biobased adipic acid market include Aemetis, Amyris (using fermentation technology), and Rennovia (through the chemical bioconversion of sugars).
- p0065 Other dicarboxylic acids with commercial interest include azelaic acid, citraconic acid, maleic acid, and crotonic acid. Biobased fermentation production routes have been proposed for azelaic acid and crotonic acid, but with very low titers and still very far from commercially relevant processes.

s0050 **9.2 Selection and Improvement of Yeast Strains**

- p0070 The enormous biodiversity of fungi and their essential role as nutrient recyclers, decomposers, mutualists, or pathogens in almost every terrestrial environment are today largely established (Hawksworth, 2001). Yeasts are unicellular fungi that are widely distributed in the natural environment and present the capacity to respond to numerous environmental stresses, such as presence of oxidative, ionic, and osmotic variations; high temperatures; nutrient limitations; starvation; high concentration of ethanol; and the presence of competing organisms. Moreover, their ability to degrade and grow in a wide range of substrates has determined their choice for commercial and industrial processes (Attfield, 1997; Querol et al., 2003; Negi et al., 2013).
- p0075 Traditionally, yeast refers to *S. cerevisiae* and its close relatives, used for alcoholic fermentation and baking. However, approximately 2000 species of yeasts belonging to 150 genera have been described so far. The genomes of over 100 yeast species have already been sequenced since the completion of the *S. cerevisiae* genome project in 1996. Presently, whole genome sequences for over a great number of *S. cerevisiae* isolates are available, revealing considerable variation among strains that might have arisen from artificial selection for novel traits through cross-hybridization (Dunn et al., 2005), horizontal gene transfer (Novo et al., 2009),

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and genomic mosaicism (Liti et al., 2009). A similar variation can be expected within all the other yeast species described to date, representing a huge reservoir of genetic diversity that may be exploited for the discovery of novel genes, entire metabolic pathways, and potentially valuable end products.

p0080 Biotechnologists usually designate all non-*S. cerevisiae* yeasts as nonconventional yeasts. What they have in common is a lower degree of fermentative overflow metabolism and a rather short history of genetic and biological characterization. The yeast *S. cerevisiae* is characterized by flourishing in extremely high sugar concentrations, most of which transformed in the by-product ethanol. The majority of natural habitats, however, do not provide such extreme substrate conditions so that most nonconventional yeasts provide alternative metabolic routes for substrate utilization, product formation, and different regulatory patterns. As an example some species of major interest are *Pichia pastoris* (syn. *Komagataella pastoris*), *Yarrowia lipolytica*, *Hansenula polymorpha* (syn. *Ogataeapara polymorpha*), *Pichia stipitis* (syn. *Scheffersomyces stipitis*), *Kluyveromyces marxianus*, or *Torulaspora delbrueckii*. [AU5]

p0085 A great number of culture collections has been developed worldwide, both public and in companies. Public collections play an important role since they offer identification facilities to meet an increasing demand for precise characterization of yeast strains of biotechnological interest, they are invaluable in the academic sphere as a source of expertise and fully authenticated material for biological research, and they provide resources to safeguard commercially important genetic variation against market failures. In addition to screening culture collections, access to biodiversity via metagenomic strategies is a promising approach to screen for improved enzymes and/or transport proteins.

s0055 9.2.1 Yeasts as Platform Organisms for Dicarboxylic Acid Production

p0090 Dicarboxylic acids, namely succinic, malic, and fumaric, are naturally occurring compounds that are found as major metabolic intermediates in most prokaryotic and eukaryotic microorganisms. The wide application of these 1,4-dicarboxylic acids as platform chemicals and the relatively few enzymatic steps required for their production have led to intensive investigation into their microbial synthesis. The yeast *S. cerevisiae* stands out as a platform microorganism for dicarboxylic acid production. Due to its role as a model organism in research, their physiology and genetics are extensively documented, and a well-developed metabolic engineering toolbox is available. In addition a huge number of genetic mutant strains are already available, such as the Euroscarf collection with around 5100 mutant strains, covering 82% of the ~6200 annotated yeast ORFs. Moreover, wild-type *S. cerevisiae* strains have GRAS (Generally Regarded As Safe) status, so that modified strains are more likely to be allowed in the production of these C4-dicarboxylic acids for food and pharmaceutical applications. [AU6]

s0060 **9.2.2 Microorganisms Producing Succinic Acid: Natural Producers Versus Metabolic Engineered Strains**

p0095 As mentioned above, several bacterial strains are known to produce considerable amounts of succinic acid under anaerobic conditions, but due to their physiological properties, they are not efficient in performing it at low pH, including facultative anaerobes (*A. succinogenes*, *Mannheimia succiniciproducens*), and strict anaerobe *A. succiniciproducens*. Many fungal [AU7] species, such as *Paecilomyces varioti* (Ling et al., 1978), *Aspergillus niger* (Papagiani, 2007), and *Penicillium simplicissimum* (Gallmetzer et al., 2002), are also found to be capable of succinate production. They can secrete succinate as a metabolic by-product under aerobic and/or anaerobic conditions, but the productivity is much lower when compared with the bacterial strains. Besides, succinate is mainly synthesized in the mitochondria, and to accumulate as an extracellular by-product, it has to cross the mitochondrial and cellular membrane (Coustou et al., 2005). Yeasts of the genus *Candida* grown on n-alkanes (Kamzolova et al., 2012), *Candida zeylanoides*, and *Candida catenulata*, grown on ethanol (Mandeva et al., 1981; Kamzolova et al., 2009), and *Y. lipolytica*, grown on rape seed oil (Kamzolova et al., 2014), have also been proposed as succinic acid producers. In addition to natural producers, many microorganisms can be metabolically engineered to produce succinate as an end product of fermentation. These are mostly model organisms since they are more prone to be genetically modified, and a completely engineered pathway is required for them to produce succinate, including *E. coli* and *Corynebacterium glutamicum*, the latter one of the few gram-positive bacteria which have been tested for succinate production.

p0100 It has been well-documented that *S. cerevisiae* achieves high concentrations of succinate to enhance the quality of wine (Wakai et al., 1980). This yeast species grows well under acidic conditions and is thus an attractive alternative as a biocatalyst for succinic acid production. Although *S. cerevisiae* wild-type strains may be used in succinic acid production, the optimization of strains via metabolic engineering for a more efficient production is currently underway, as mentioned earlier.

s0065 **9.2.3 Succinate Formation Pathways**

p0105 The production of organic acids begins in yeast via glycolysis, differing then downstream of pyruvate formation. From pyruvate, three pathways are possible for the succinate formation: via oxidative tricarboxylic acid (TCA) cycle, via reductive branch of TCA cycle, or via oxidative pathway of the glyoxylate cycle.

s0070 *Oxidative tricarboxylic acid cycle*

p0110 Under aerobic conditions, succinate is an intermediate metabolite of both the oxidative TCA cycle and the glyoxylate shunt. In the TCA cycle (Fig. 9.1A), acetyl-CoA generated from pyruvate is joined with oxaloacetate, and via the action of citrate synthase (encoded by gene

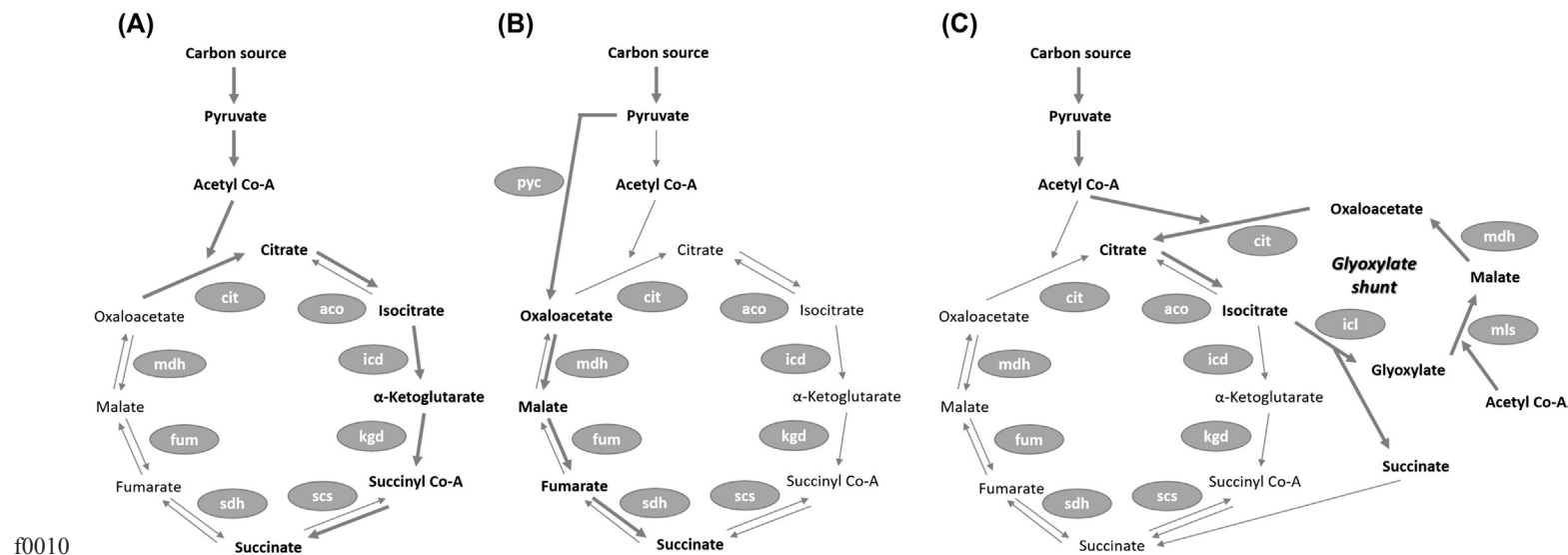


Figure 9.1

Main succinate production metabolic pathways in *Saccharomyces cerevisiae*: (A) oxidative branch of TCA cycle; (B) reductive branch of TCA cycle; (C) glyoxylate pathway. Not all enzymatic steps are shown. Abbreviations used for enzymes are: *aco*, aconitase; *cit*, citrate synthase; *fmds*, fumarate reductase; *fum*, fumarase; *icd*, isocitrate dehydrogenase; *icl*, isocitrate lyase; *kgd*, α-ketoglutarate dehydrogenase; *scs*, succinyl-coenzyme A synthetase; *mdh*, malate dehydrogenase; *mls*, malate synthase; *pyc*, phosphoenol pyruvate carboxylase; *sdh*, succinate dehydrogenase.

CIT), will form citrate. Aconitase (*ACO1*) will then convert citrate to isocitrate, which will be transformed in α -ketoglutarate by isocitrate dehydrogenase (encoded by genes *IDH* and *IDP1*). The molecule of α -ketoglutarate will then be decarboxylated by α -ketoglutarate dehydrogenase and form succinyl-CoA, which finally is converted to succinate by succinyl-CoA synthetase (encoded by gene *LCSI*). If the TCA cycle continues, enzyme succinate dehydrogenase (encoded by genes of the complex *SDH*) will convert succinate to fumarate. Therefore, one key step of metabolic engineering strategies is the inactivation of *SDH* genes in order to stop this conversion to fumarate, and in this way, allowing the accumulation of succinate in the cell.

s0075 *Reductive branch of tricarboxylic acid cycle*

p0115 Under anaerobic conditions, the reductive pathway of TCA cycle is activated, since succinate is the H-acceptor instead of oxygen, and pyruvate, originated from glycolysis, is converted to oxaloacetate, malate, fumarate, and then succinate (Fig. 9.1B). This pathway, from phosphoenolpyruvate (which precedes pyruvate in glycolysis) to succinate, requires 2 mol of NADH per mole of succinate produced, which represents a maximum theoretical yield of two molecules of succinate for every glucose molecule, since each molecule of glucose can provide only two molecules of NADH through glycolysis. Therefore, the redirection of the carbon flux only to the anaerobic fermentation pathway is energetically unfavorable, has a theoretical yield higher than the oxidative direction or the glyoxylate shunt (1.71 mol per mole of glucose), and results in net CO₂ fixation. The reductive pathway can be divided in the following steps: (1) pyruvate carboxylation, in which pyruvate is converted in oxaloacetate, a reaction performed by the enzyme pyruvate carboxylase, encoded by gene *PYC*; (2) oxaloacetate reduction to malate, via the action of malate dehydrogenase, encoded by gene *MDH*; (3) translation of malate to fumarate, under the action of fumarase (encoded by *FUMR*); and (4) fumarate reduction performed by fumarate reductase, encoded by genes *FRDS1* and *OSHI*. Yan et al. (2014) reviewed the main obstacles of reductive TCA pathway for succinic acid production: (1) yeast fumarase (*FUM*) only converts fumarate to malate without the possibility to revert the process to fumarate production; (2) fumarate reductase, the key enzyme involved in the reductive production of succinate (Arikawa et al., 1999), coded by genes *FRDS1* (cytosol) and *OSMI* (mitochondria) (Fig. 9.1B), are only produced under anaerobic conditions; and (3) a high amount of NADH is consumed by this branch (2 mol of NADH per each mole of succinic acid formed).

s0080 *Oxidative pathway of glyoxylate cycle*

p0120 The glyoxylate shunt is also a possible pathway to produce succinic acid in aerobic conditions. One large part of the glyoxylate cycle is similar to the TCA cycle, sharing three of the five enzymes associated with the TCA cycle and many of its intermediate steps. The main variations are related with the conversion of isocitrate into glyoxylate and succinate by isocitrate lyase (encoded by gene *ICLI*). Then glyoxylate is converted into malate by the action of malate synthase (Fig. 9.1C). Succinate production via glyoxylate cycle instead of

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TCA cycle has the advantage of avoiding mitochondrial transport, since it occurs in the cytosol, and also leads to less carbon loss due to only one decarboxylation step. When compared to the reductive cycle, the glyoxylate shunt has a lower theoretical yield but provides ATP for maintenance and active transport processes (Raab and Lang, 2011).

p0125 Yet another alternative to produce succinate is the combined use of both the reductive TCA cycle and glyoxylate shunt by joining the oxidative and reductive route, providing in this way an even redox balance, a higher maximum succinate yield, and a fixation of CO₂ instead of its release (reviewed in Raab and Lang, 2011). However, its implementation will be a challenge in the following years, since it requires that oxidative and fermentative metabolism run simultaneously in yeasts. This strategy was already successfully accomplished in *E. coli* strains, with an increase of the theoretical yield (Rezaei et al., 2015; Vemuri et al., 2002a,b).

s0085 9.2.4 Metabolic Engineering Strategies for Biotechnological Production of SA

[AU8]

s0090 *Saccharomyces cerevisiae*

p0130 Over the past two decades, research has focused efforts in the biotechnological succinate production process, in particular using metabolic engineering. Succinate is not normally produced at high levels in *S. cerevisiae*, but there are many reasons for choosing this yeast as a microbial cell factory for platform chemicals, including the easiness of genetic manipulation, the extensive library of genetic engineering tools and a very well-annotated genome, their ability to grow both anaerobically and aerobically, and the proven ability of being used at industrial scale.

p0135 Different strategies have been used to manipulate yeast strains to an enhanced production of succinic acid, as reviewed in Cheng et al. (2013). The challenge in metabolic engineering of *S. cerevisiae* for the efficient production of succinic acid involves at least three levels: (1) elimination of alcoholic fermentation, which occurs irrespective of the availability of oxygen; (2) engineering fast and efficient metabolic pathways that link the high-capacity glycolytic pathway to the TCA cycle, taking into account NADH and ATP constraints; and (3) engineering of product export.

p0140 When increased succinate production is the goal, ethanol production should be avoided, since it will change the carbon flux from pyruvate to ethanol, leading to carbon loss in the process. However, even under fully aerobic conditions, *S. cerevisiae* strains are intrinsically associated to alcoholic fermentation, and it is hard to redirect the flux from pyruvate to organic acids production, instead of ethanol formation (Abbott et al., 2009). The pathway that converts pyruvate into ethanol involves only two targets for metabolic engineering via gene deletion: pyruvate decarboxylase and alcohol dehydrogenase. In the first step to produce ethanol, pyruvate is decarboxylated to acetaldehyde in an irreversible reaction catalyzed by pyruvate decarboxylase. *Saccharomyces cerevisiae* contains three structural genes that encode functional pyruvate decarboxylase isozymes, *PDC1*, 5, and 6. Although deletion of all three genes completely eliminates alcoholic fermentation, pyruvate decarboxylase-negative (Pdc⁻) strains

grow poorly in complex media and fail to grow on glucose as the sole carbon source. This inability is due to the need of producing cytosolic acetyl-CoA, an essential precursor for lysine and lipid synthesis, and the fact that in *S. cerevisiae* the mitochondrial acetyl-CoA, produced via pyruvate dehydrogenase, cannot be transported to the cytosol. Thus, the requirement for C2 compounds (to overcome cytosolic acetyl-CoA need) and the high glucose sensitivity represents major impediments for the use of Pdc⁻ strains for succinic acid production. However, by applying evolutionary engineering, these two limitations could be overcome, and Pdc⁻ *S. cerevisiae* isolates are available showing high pyruvate concentrations, up to 135 g/L at a yield of 0.54 g/(g glucose) (van Maris et al., 2004). The second step in the conversion of pyruvate to ethanol is the reduction of acetaldehyde to ethanol through the action of alcohol dehydrogenase. *Saccharomyces cerevisiae* contains four alcohol dehydrogenase (*ADH1* to *ADH4*) structural genes. Strains deleted in these genes were observed to grow poorly, mainly due to intracellular accumulation of glycerol and of toxic acetaldehyde (Drewke et al., 1990). Thus, to block ethanol formation, the best alternative is the elimination of pyruvate decarboxylase activity. Since these *S. cerevisiae* strains produced high titers and yields of pyruvate, Pdc⁻ strains have a great potential for the production of organic acids, especially those for which pyruvate is an intermediate metabolite (Flikweert et al., 1996; Flikweert and Johannes, 1997).

p0145 One of the first attempts to improve succinate production was reported by Arikawa et al. (1999) using sake yeast strains with TCA cycle gene deletions (Table 9.2). The simultaneous deletion of *SDH1* and *FUM1* led to 2.7-fold higher production of succinate in comparison with wild-type strains. However, these enhancements were not observed in the anaerobic

t0015 **Table 9.2: Main Metabolic Engineering Strategies to Improve Succinate Production in Different Yeast Species.**

Strain	Genotype	Succinate Production	References
<i>Saccharomyces cerevisiae</i>			
Kura	$\Delta SDH1 \Delta FUM1$	2.32 g/L	Arikawa et al. (1999)
AH22ura3	$\Delta SDH1, \Delta SHD2, \Delta IDH1, \Delta IDP1$	3.62 g/L, 0.11 mol/mol glucose	Raab et al. (2010)
8D	$\Delta SDH, \Delta SER3/SER33$, overexpression of native <i>ICL1</i>	0.9 g/L	Otero et al. (2013)
BY4741	$\Delta DIC1$	0.23 g/L	Agren et al. (2013)
PMCFF	$\Delta FUM1, \Delta PDC, \Delta GPD1$	8.09 g/L	Yan et al. (2014)
S149sdh12	$\Delta SDH1, \Delta SHD2$, expression of <i>MAE1</i> gene	2.36 C-mol yield	Ito et al. (2014)
<i>Yarrowia lipolytica</i>			
Y-3312	$\Delta SDH1, \Delta SHD2$	45 g/L	Yuzbashev et al. (2010)
<i>Issachenkia orientalis</i>			
SD108	$\Delta PYC, \Delta AMDH, \Delta FUMR, \Delta FRD$	11.63 g/L	Xiao et al. (2014)

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conditions of sake fermentation. Another metabolic engineering strategy for the oxidative production of succinic acid was the quadruple gene deletion (*SDH1*, *SDH2*, *IDH1*, *IDP1*) [AU10] described by Raab et al. (2010). In glucose-grown shake-flask cultures, the mutant strain produced a titer of 3.62 g/L (factor 4.8 as compared to wild-type), the highest obtained for yeast until then. Multigene deletion followed by directed evolution was used by Otero et al. (2013) to select a succinate producer mutant. The strategy included deletion of *SDH3* and interruption of glycolysis derived serine by deletion of 3-phosphoglycerate dehydrogenase (*SER3/SER33*) and overexpression of native *ICL1*. The mutant strain presented a 43-fold increase in succinate yield on biomass compared to the reference strain. A modified pathway for succinate production was established by the deletion of genes *FUM1*, *PDC*, *GPD1*, and was reported by Yan et al. (2014). The authors obtained a succinate titer of 8.09 g/L, which was further improved to 9.98 g/L through regulation of biotin and urea levels, and to 12.97 g/L through optimal CO₂ conditions in a bioreactor. Another attempt to construct *S. cerevisiae* strains with increased succinate production involved the disruption of *SDH1* and *SDH2* genes, together with the expression of *Schizosaccharomyces pombe* malic acid transporter *MAE1*. [AU11] The expression of the transporter to export intracellular acid outside of the cell successfully improved succinic acid production (Ito et al., 2014). Table 9.2 summarizes the main metabolic engineering strategies to improve succinate production in *S. cerevisiae* and other yeast species.

s0095 Other yeasts

- p0150 Yuzbashev et al. (2010) proposed a new strategy for construction of an aerobic eukaryotic succinic acid producer, based on the yeast *Y. lipolytica*, with a deletion in the gene coding for one of the succinate dehydrogenase subunits. Firstly, an original in vitro mutagenesis-based approach was proposed to construct strains with temperature-sensitive mutations in the *SDH1* gene. These mutants were used to optimize the composition of the media for selection of transformant strains with the deletion in the *Y. lipolytica* *SDH2* gene. Although the defects of each succinate dehydrogenase subunit prevented the growth on glucose, the mutant strains grew on glycerol and produced succinate in the presence of the buffering agent CaCO₃. Subsequent selection of the strain with deleted *SDH2* gene for increased viability allowed the construction of a strain capable of accumulating succinate at the level of more than 45 g/L in shaking flasks with buffering and more than 17 g/L without buffering agent.
- p0155 Due to its ability to grow at low pH and high tolerance to multiple organic acids, the yeast *Issatchenkia orientalis* has also been used as a microbial platform for succinic acid production (Xiao et al., 2004). Based on the fact that the reductive TCA cycle gives the highest theoretical yield on glucose (1.31 g/g), genes from this metabolic pathway were selected as targets for metabolic engineering. The metabolic strategy designed involved the assembly and integration of four genes (*PYC*, *MDH*, *FUMR*, and *FRD*) from the reductive TCA pathway in the genome of a uracil auxotroph strain of *I. orientalis* (SD108). To obtain high transcription [AU12]

levels, the genes were individually cloned to downstream of strong promoters, and to avoid the potential issue with succinic acid being exported across the inner membranes (eg, from mitochondria to cytoplasm), potential signal peptides were removed so that the enzymes would be expressed in the cytoplasm. In batch cultures using shake flasks, the resulting metabolic engineered strain (*IoΔura3* + SA) was able to consume all glucose (100 g/L) and produce 11.63 g/L of succinic acid, after 110 h of growth, while strain *IoΔura3* + *ura3* consumed 99.29 ± 0.08 g/L glucose and yielded 1.43 ± 0.04 g/L succinic acid during the same period.

s0100 **9.3 Production of Biobased Dicarboxylic Acids**

s0105 **9.3.1 From Strain Isolation to Initial Physiological Studies**

p0160 When developing a process for industrial implementation, the central concern is providing specific conditions for the microorganism perform at highest productivity at each stage of the bioprocess. To do so, the process has to be tuned to the specific requirements of the microorganism being used, and thus, the prior knowledge of its physiology and dynamic behavior in culture is crucial. The candidate strains may be well-known strains obtained from culture collections, genetically modified organisms after metabolic engineering optimization rounds or the introduction of heterologous pathways, or new isolates naturally occurring in nature.

p0165 Given the diversity of microorganisms naturally occurring in nature, the isolation of a desired population of microorganisms from natural sources is a time-consuming task, which could culminate in the isolation of hundreds of potentially desired strains. When a large number of isolates needs to be tested, high-throughput phenotypic screening can be used to carry out experiments in parallel rather than sequentially. In general, many variables can be tested at once, namely, different strains, carbon and nitrogen sources, nutrient concentrations, pH, and temperature. A simple yet powerful way to do so involves, for example, the use of deep-well microplates coupled with optical density analysis in microplate readers (biomass concentration) and HPLC analysis with microplate-compatible autosamplers (for analysis of metabolites). The different well geometries and orbital agitation conditions further enable to modulate the mass transfer efficiency and thus aeration (Duetz et al., 2000), which can be varied from microaerobic to fully aerobic conditions. By cultivating the cells in static deep-well microtiter plates, anaerobiosis is achieved. More sophisticated multiplexed systems exist, which allow monitoring online the variation of parameters, such as the optical density, the dissolved oxygen concentration, and the pH, but the high cost of these systems and the time-consuming data analysis of the various time profiles recorded has prevented their widespread use.

[AU14]

p0170 Initially, the screening should be performed in a medium known to enable sustained growth of the candidate microorganisms and to favor the production of the target compound. For example, in the case of the production of succinic acid from yeasts, de Klerk (2010) described

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a culture medium that simulates grape must, used for the isolation of succinic acid producing wine yeasts. According to the author, succinic acid production by fermenting yeasts will be favored by moderate to high fermentation temperatures in must with a nicotinic acid and/or nicotinamide deficiency, high sugar content, moderate amounts of metabolically available nitrogen, presence of flavonoids, and large supplies of unsaturated long-chain fatty acids. Using a number of 96 deep-well microtiter plates, a fast screening is achieved by growing a different isolate in each well, especially if some degree of automation is available. The screening should be focused on parameters relevant for the envisaged larger scale production, such as cell growth kinetics and product concentration production. When using the high-throughput system, sampling during the incubation period is limited, since the volume of culture in each well ranges from 1 to 2 mL. For that reason, samples should be only collected in the end of the incubation period or use multiple replicate microtiter plates, which allows characterization of each culture at different incubation periods by simply removing one of the replicas from the incubator at a given point. This array of experiments will typically allow for the selection of a significantly narrowed range of candidate strains, which are then brought to shake-flask experiments, allowing for more detailed physiological studies. Again, the mass transfer during the specific culture conditions will determine the aeration efficiency of the culture. As explained above, this will have major effects on the metabolism and particularly on succinic acid production. The aeration efficiency will depend on the stirring speed, the ratio of volume of liquid versus total flask volume, and on particular features the shake flask may be equipped with (eg, baffles).

p0175 In the case of the production of succinic acid using yeast strains, this screening activity be based on measurements of parameters such as: biomass concentration, residual main carbon source concentration, concentration of succinic acid, concentration of other carboxylic acids, concentration of ethanol. If various conditions are tested, such as different fermentation times and different agitation/aeration regimes, preliminary, but important, information will be derived that will enable designing fermentation strategies to be implemented in fully controlled bioreactors, simulating the large-scale equipment.

s0110 9.3.2 Linking Strain Physiology to Bioreaction Conditions

p0180 As mentioned above, the way carbon is processed through the central carbon pathway depends not only on the carbon source concentration, but also on the availability of oxygen. When the carbon source and oxygen are available without limitation in *Saccharomyces*, the carbon flux to the TCA cycle will be split at the pyruvate node, due to intrinsic limitation of the respiratory capacity of the yeast (Crabtree effect). As result, part of the carbon source will be diverted into unwanted compounds such as ethanol and eventually acetate, thus diminishing the yield of the product of interest but also inhibiting growth if high concentrations of ethanol or acetate are reached. This will happen not only with *Saccharomyces* strains,

but with all Crabtree-positive yeasts. These conditions are typically those occurring when using a batch fermentation strategy in which all components of the fermentation medium are present at the time of inoculation of the bioreactor and the additions to the bioreactor are limited to those required to control the dissolved oxygen concentration (air) and the pH (acid and/or base). The absence of carbon or oxygen limitation results in the most energetically efficient use of carbon source (maximizes the generation of ATP), but mainly targets metabolism to biomass production with generation of CO₂. Regarding succinic acid, although it is produced in the TCA cycle, it is hardly accumulated and readily converted to fumarate and beyond to complete the cycle. Further, the enzymes involved in the glyoxylate cycle are known to be repressed when high concentrations of glucose are present. One way to try to circumvent this diversion of carbon from the target pathway is the adoption of a fed-batch strategy in which a small amount of main carbon source is present in the culture medium at the time of inoculation and the carbon source is gradually fed throughout the culture, ex. as a concentrated glucose solution, to supply, ideally, the nutrient at the exact rate at which it is being consumed by the culture, thereby keeping a constant concentration in the bioreactor below the threshold of the respiratory capacity of the yeast. The purpose of using a high concentration of sugar is to reduce the dilution of the culture when the solution is added by minimizing the volume of solution that needs to be added to maintain the desired concentration in the bioreactor. This strategy also allows supplying a much higher total amount of nutrient than that possible in a batch fermentation without causing substrate inhibition or the formation of high concentrations of by-products, some of which potentially inhibit growth. Further, when using complex raw materials, particularly complex by-products of agroforestry industries, the growth inhibitors that may be present on those materials will be highly diluted at the initial stages of the fermentation, and their concentrations will only become meaningful when a robust high-cell density culture has been developed, thus more able to cope with those inhibitors. In the case of yeast, even if some ethanol or acetic acid are produced, these can be used as substrate as soon as the main carbon source is limiting or absent in aerobic conditions. It has been shown that these are essentially processed through the glyoxylate cycle, which generates succinate and malate. When the main carbon source is mainly processed through a fermentative pathway due to oxygen limitation, ethanol is formed. However, part of it could be directed to the reductive direction of the TCA cycle, although this pathway is much less active than the ethanol production pathway due to the kinetic parameters of the involved enzymes for the reductive route of TCA cycle and due to the resulting redox imbalance. When sufficiently high concentrations of CO₂ are present, the equilibrium of the conversion of isocitrate to α -ketoglutarate and of α -ketoglutarate to succinyl-CoA will be affected, and the conversion of isocitrate to succinate through the glyoxylate cycle will be favored. Additionally, the conversion of pyruvate to oxaloacetate and the cycling of TCA in the reductive direction will also be favored. These carboxylation reactions can be favored by sparging CO₂ or by adding carbonate salts in the fermentation medium.

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p0185 Since succinic acid is a member of the core carbon metabolism cycle, in normal circumstances, yeasts will not accumulate it, any of the other dicarboxylic acids upstream, or downstream succinic acid in that cycle. Another way to foster the accumulation of the intermediate components of the TCA cycle would be to restrict biomass formation. Indeed, the TCA cycle intermediates oxaloacetate and α -ketoglutarate are precursors of biomass components, namely amino acids, and provide carbon diversions from the target product. In order to limit the diversion of carbon and TCA intermediates to biomass formation, restrictions in the supply of, for example, nitrogen, can be imposed. Without nitrogen, those precursors cannot be converted into nitrogen-containing molecules, such as amino acids, the building blocks of proteins, and therefore their diversion from the TCA cycle will be diminished. One very effective way to supply a fermentation process with the required nitrogen for biomass buildup is the control of the pH with ammonium hydroxide, balancing the pH lowering effect of the production and accumulation of organic acids, namely acetic acid. If the pH control agent is shifted from ammonium hydroxide to sodium hydroxide, less nitrogen will be available, eventually creating nitrogen limitation, thus favoring the reactions not involving biomass formation.

s0115 9.3.3 Requirements for Scale-Up Toward Commercial Operation

p0190 Various technical issues must be tackled in order to deploy a process that can adequately perform at commercial scale. First and foremost, the process must be robust enough in order to be replicated without significant performance deviations between production batches. This implies that the slight variations in raw material quality should not impact significantly on the process, that the implemented controls are able to maintain the process within the desired operating windows, and that the microorganism is stable during the batch, and cultures in the cell banks are adequately standardized. This is particularly important when genetically modified strains are used, since they are normally under a more demanding, stressful, and energy-demanding physiological state than their nongenetically modified counterparts, which, if present, tend to dominate the culture.

p0195 Although scale-up of a fermentation-based process may seem a simple multiplication factor of all aspects of the process, it is actually one of the most challenging tasks in bioprocess engineering. Indeed, the larger the scale of a bioreactor, the further will it be from ideal behavior. For example, in larger bioreactors, mass transfer issues become highly relevant since concentration and temperature gradients become significant, which originate diverse microenvironments within the bioreactor that can dramatically affect the metabolism and cell viability. For example, upon feeding a concentrated sugar solution to the bioreactor, the cells on the region in which the sugar solution drops will be subjected to a very high sugar concentration and therefore may trigger the diversion of the metabolism toward the generation of by-products (eg ethanol in *S. cerevisiae* or acetic acid in *E. coli*). Similarly, cells that at a certain instant flow past the air

sparger will be subjected to a very high dissolved oxygen concentration, while those that at the same instant are flowing at the top of the fermenter will be in an environment with a much lower and eventually limiting oxygen concentration, with evident differences in their metabolic state. These heterogeneities can therefore lead to decreases in productivity and yields as compared to the same process as implemented at small-scale in conditions close to ideal mixing. These scale-up issues are particularly important for aerobic processes (gradients in oxygen concentration) and in fed-batch operation, in which the continuous or periodic addition of solutions, often highly concentrated, is performed.

s0120 **9.4 Downstream Processing of Biobased Dicarboxylic Acids**

s0125 **9.4.1 General Steps in Downstream Processing**

p0200 Once produced by fermentation, the dicarboxylic acids in the fermentation broth need to be recovered and purified to meet the specifications of the final product. This process is called downstream processing (DSP) and includes several unit operations. It may comprise all or some of the following steps: cell disruption, separation, recovery, concentration, purification, product stabilization, and formulation of a target molecule (Table 9.3). The DSP also includes consideration of side-streams and reuse of effluents. This section gives an overview of general steps in DSP of a broth produced by submerged fermentation.

p0205 The choice of unit operations for a specific DSP sequence has to consider the final product application, as different specifications regarding purity, biological activity, molecular weight, etc., might be required. The understanding of the chemical and physical properties of the target molecule and the specific process is a prerequisite for designing an adequate DSP. As dicarboxylic acids are excreted to the fermentation broth, no cell disruption is required. For separation of cells from the fermentation broth, the common solid/liquid separation technologies are sedimentation or centrifugation, based on the difference in density/gravity, or filtration, based on the difference in particle size, typically microfiltration. After cell removal, the dicarboxylic acids need to be recovered from the fermentation broth. The cells may be recirculated back into the fermentation process or discarded. The following recovery process aims at the crude separation of soluble components (eg, proteins, sugars), which are different in their physical and chemical properties compared to the target molecule. Here, the separation may be based on differences in molecular size in which filtration is again used, typically through ultrafiltration or nanofiltration membranes; differences in solubility, through precipitation processes; and differences in chemistry, for example, through solvent extraction or reactive extraction. Between steps, it is often desirable to reduce the amount of liquid material, and therefore a concentration step may be used for the purpose. In fact, in submerged fermentations, the fermentation broth may contain up to 95% of water. A simple concentration process is the removal of water or solvents via evaporation. For heat-sensitive metabolites

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Table 9.3: General Steps in Downstream Processing, Highlighting Common Principles and Unit Operations as well as the Respective Desired Outcome of Each Step.

Step	Principle	Unit Operation	Aim
Cell disruption	Mechanical	High pressure, homogenization, elongation and shear-stress	Disrupted cells released the target molecule
	Chemical	Addition of lytic agents, eg, solvents, surfactants, alkalis	
	Physical	Heat, sonication, freezing, osmotic shock	
	Biological	Specific enzymes	
Separation (liquid/solid)	Density/gravity	Centrifugation, sedimentation	Particle-free liquid or solid containing the target molecule depending on solubility in fermentation broth
	Particle size	Filtration	
Recovery and concentration	Solubility	Precipitation	Removal of components not similar to target molecule, eg, proteins, sugars, water >Different chemical/physical properties
	Molecular size	Membrane filtration	
	Chemical	Solvent extraction, reactive extraction	
Purification	Physical	Evaporation of water/solvent	Removal of components similar to target molecule, eg, salts, other organic acids >Similar chemical/physical properties
	Solubility	Crystallization	
	Affinity	Chromatography, adsorption	
	Ionic charge	Electrodialysis	
Product stabilization & formulation	Drying	Spray drying, freeze drying	Final product formulation
	Agglomeration	Fluidized bed drying	
	Sterilization	Heating	
	Blending/packing	Ingredients for specific product properties	

(like proteins), evaporation under reduced pressure or membrane process can also be applied. However, the energy-demanding water removal is often a later-stage step after less demanding operations allowed for a preliminary quantity reduction of processed streams. Subsequent purification results in the removal of components (eg, salts, other organic acids), which are difficult to separate because of their similar physical and chemical properties compared to the target molecule. Steps for separation of similar molecules usually contribute the most to the cost of DSP due to high equipment and operating costs. Depending on the type of molecule and final product requirements, further purification steps can include crystallization, based on differences of solubility; chromatography and adsorption, based on different affinity toward a solid phase; and electrodialysis or ion-exchange chromatography, mainly based on differences in charges of molecules. After sufficient purification, the final product may need to be stabilized for shipping, storage, and sale. The most common principle here is drying the product.

Depending on sensitivity of the product, different unit operations are available such as freeze-drying, spray drying, fluidized bed drying, etc. Agglomeration, sterilization, or blending might also be required for some final product formulations.

s0130 **9.4.2 Factors Influencing Downstream Processing**

- p0210 Downstream processing is an integral part of the entire production process and is influenced by many factors in addition to the bioprocess itself, including the raw materials used, the specifications of the final product or the operations that may follow directly after DSP, such as formulation or direct in situ use, such as polymerization. Further, in some instances, at least part of the DSP sequence may be closely integrated with the fermentation step, whereby in situ separation of products can be performed. DSP make up a significant part of the total production costs, ranging between 50% and 90% of the total production process.
- p0215 The type of microorganism (filamentous fungi, yeast, bacteria), product (intra-/extracellular, chemical and physical properties), fermentation substrate, fermentation process (submerged, solid state), and target application (purity specifications) influence the decision for the specific choice of DSP. In most cases, different combinations of steps and different unit operations have to be used. As mentioned earlier, various by-products may arise from succinic acid fermentation, which have similar structures and properties, including malic acid and lactic acid, and their selective separation has a significant impact on DSP costs. The use of recombinant strains may allow for less production of metabolic by-products.
- p0220 Bacterial fermentation for the production of succinic acid is usually performed at neutral pH. Due to constant release of organic acids, the pH decreases during fermentation, and the effect of product inhibition usually occurs, unless pH control is implemented, normally through the use of calcium, magnesium, sodium or potassium hydroxides, carbonates, or ammonium salts
- [AU16] or simply. This leads to the production of a succinate salt, which then needs to be converted into succinic acid through acidification with sulfuric acid, generating gypsum as a by-product. Yeasts are more tolerant to acidic conditions and may be used in processes at significantly lower pH. At a pH below 3, many carboxylic acids are predominantly present in their undissociated form (eg, succinic acid $pK_{a1} = 4.2$, and $pK_{a2} = 5.6$ at 25°C (Dean, 1999)). In this case, the requirements of pH-controlling elements are smaller, resulting in lower consumption of chemicals, and less waste produced since the undissociated free acid is directly recovered (Abbott et al., 2009).
- p0225 Generally, carbon sources are typically provided by fermentation substrates containing easily fermentable carbohydrates such as mono- or disaccharides. An increasing diversity of low-cost but complex raw materials such as agro-industrial wastes, are being considered by the chemical industry as it replaces its petroleum based production processes with industrially biobased fermentation processes. Pretreatment of material (eg, digestion, removal of inhibitors) becomes necessary, allowing for a cleaner broth, better fermentation results, and

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consequently lower DSP costs. Yet, fermentation based on these alternative raw materials includes a more diverse initial mix of complex components, which need to be separated after the fermentation in order to meet product specifications.

p0230 The choice of unit operation for the DSP is also influenced by the intended application of the final product. For instance, some applications require large amounts of the target product in lower quality, while others require smaller amounts of high quality and biological active material. Further, the nature of contaminants can be determinant. In general, for common applications of succinic acid a high purity is required (>98%). If the product is intended to be used in the production of polymers, such as PBS, it can tolerate small amounts of other dicarboxylic acids, which still have two functional groups sustaining the chain extension during the polymerization process. Conversely, even minute amounts of monocarboxylic acids will be detrimental to the final product, since they only have one functional group and, as such, will act as polymerization terminators.

p0235 The type of target metabolite and thus its chemical and physical characteristics have the highest impact on designing the DSP. This mainly includes polarity, acid dissociation constant, sensitivity, solubility, etc. In summary, it can be said that designing an effective DSP is very specific and needs to evaluate the chemical and physical properties of the target molecule, the requirements of desired application, and the reuse of effluents and side products.

s0135 9.4.3 State of the Art DSP for Dicarboxylic Acids and New Trends

s0140 Precipitation

p0240 State of the art recovery processes of dicarboxylic acids take advantage of precipitation technologies that are currently industrially applied, ie, in the production and purification of lactic, citric, and acetic acids (Kurzrock and Weuster-Botz, 2010; Yang et al., 2007). Thus, equipment, technology, and infrastructure can often be adapted or even directly employed (Cheng et al., 2012a). In the case of succinic acid, biomass is removed from the carboxylate-rich fermentation broth, which is then acidified. The control of pH during fermentation through the addition of Ca^{2+} or $\text{Ca}(\text{OH})_2$ has the disadvantage of the lower solubility of the calcium carboxylate, which may lead to the precipitation of calcium salts already during fermentation. This is the reason why also other pH controlling elements, like sodium and magnesium hydroxide, were investigated (De Haan et al., 2013). The precipitation may be facilitated by cooling the fermentation broth, and the resulting succinates can be separated via filtration or centrifugation and then converted to succinic acid by adding concentrated H_2SO_4 . Further purification steps may include active carbon absorption or ion exchange followed by concentration and crystallization via evaporation. Disadvantages of this process are the high dosages of $\text{Ca}(\text{OH})_2$, or CaO and H_2SO_4 , which cannot be reused. Additionally, the undesirable by-product, CaSO_4 (equimolar to succinic acid), is of low economic value

(Datta, 1992; Datta et al., 1992; Berglund et al., 1999). A yield of 91% (Datta et al., 1992) and a purity of 94.2% can be achieved. The precipitation of ammonium carboxylates has the advantage of possible regeneration of reagents (including sulfuric acid and ammonia through thermal cracking). Thus, lower amounts of waste by-products accumulate since the recovered acid and base can be reused during the fermentation and the DSP, while achieving higher recovery rates (yield 93.3%) (Yedur et al., 2001). Disadvantages include the low selectivity of precipitation, high energetic costs, and the need for durable equipment that is able to withstand the severe conditions of temperature and acidity during recycling of used reagents.

s0145 *Extraction*

p0245 The cell-free fermentation broth is an aqueous phase from which a specific dicarboxylic acid needs to be selectively extracted into another liquid phase (aqueous–solvent or aqueous–aqueous), immiscible with the fermentation broth. Each target molecule has a special distribution coefficient also depending on the parameters of extraction, such as pH, temperature, ionic strength, and type of solvent (Ottens et al., 2006). The distribution coefficient is defined as the ratio of the concentration of the target acid between two immiscible phases at equilibrium. The distribution coefficient should be high for the targeted product and low for other substances in the fermentation broth, indicating preferential solubility in the solvent and hence good separation, while a lower distribution coefficient would require multistage extraction and large amounts of solvent. Classical solvent extraction using, for example, alcohols, ketones, esters, or aliphatic hydrocarbons is not effective in the DSP of dicarboxylic acids because of low distribution coefficient (<1), resulting in low yield and purity. One way to increase the distribution coefficient is adding a reactive compound, eg, an amine, through a process called reactive extraction (Yang et al., 2013; Kertes and King, 1986). The chosen reactive compound should ideally have low water solubility, high distribution coefficient for the target dicarboxylic acid, and low distribution coefficient for other organic acids or impurities (Thongchul, 2013). Generally, a reactive extraction system comprises the steps of (A) reactive extraction followed by (B) back extraction as shown in Fig. 9.2, in this particular case using a displacer. The entire process also includes prior cell separation as well as subsequent concentration, crystallization, and drying.

p0250 In the reactive extraction stage, the cell-free fermentation broth with the target acid is mixed with the organic phase containing the reactive compound. Long-chain aliphatic amines have been used successfully as reactive compounds for dicarboxylic acids (Hong and Hong, 2004; Kurzrock and Weuster-Botz, 2010; Kurzrock, 2010; Lee et al., 2008, Tung and King, 1994). At the interphase of aqueous and solvent phase, the amine forms a complex with the acid. The acid–amine complex is solvated in the organic phase. Some amines benefit from the presence of a diluent, which reduces the corrosiveness and high viscosity of the amines, while also

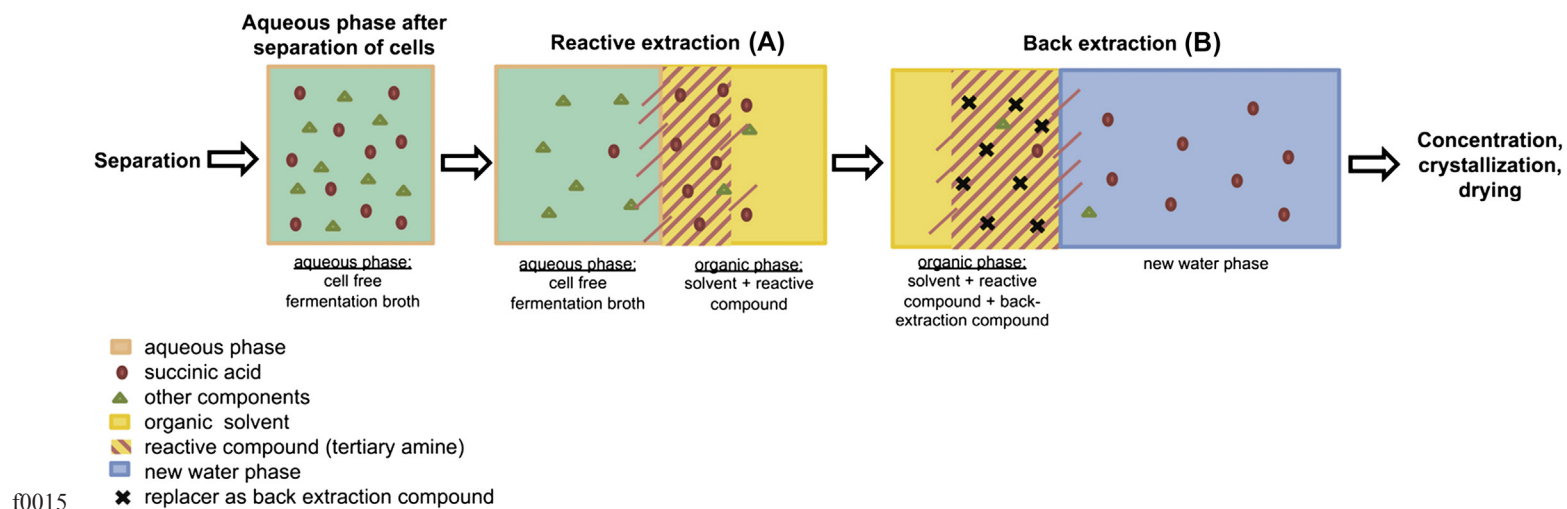


Figure 9.2

Schematic principle and processing steps during (A) reactive extraction and (B) back extraction system with displacer as back extraction compound.

- having an effect on the extractive capabilities (Kurzrock and Weuster-Botz, 2011; Huh et al., 2006; Tamada and King, 1990). Active solvents (such as 1-butanol, 1-hexanol, and 1-octanol) are recommended to improve the solvation of hydrophilic organic acid by hydrophobic long-chain aliphatic amines (Yang et al., 2013; Thongchul, 2013) by stabilizing the acid–amine complex, while inert solvents (such as alkanes) act solely as solvating media (Kurzrock and Weuster-Botz, 2011). Generally, the efficiency of extraction with aliphatic amines depends largely on the pH, temperature, by-products in the fermentation broth, the concentration of target acid, the properties of the solvent and amine, the loading ratio, and distribution coefficient. In a similar fashion, other extractive elements can be employed, the organophosphines. These substances, which are strong Lewis bases, were also tested for the recovery of succinic acid, albeit no application in recovery from fermentation broths has been reported. After the reactive extraction stage, it is necessary to recover the dicarboxylic acid, which is solvated in the organic phase as a nonvolatile acid–amine complex, and to recycle the costly amines and the organic phase. Back extraction is often suggested as the best alternative. It is based on a change in distribution coefficient between the organic phase and a new water phase, depending on operating variables such as a change of solvent, swing in temperature, and/or disintegration of the acid–amine complex by shift in pH or addition of a displacer, typically a stronger acid or base (eg, Yabannavar and Wang, 1991; Lee et al., 2008; [AU17] Keshav and Wasewar, 2010; Kurzrock, 2010; Sadaka and Garcia, 1998). Typically, a combination of these methods yields better results. After back extraction, the solvent is separated from the initial fermentation broth, which can be recirculated into the fermentation process. The advantage is the backflow of nutrients and remaining dicarboxylic acid, which was not completely extracted. However, the recirculate might still contain residues of solvent, which should be biocompatible in order to prevent inhibition of the microorganisms. Overall, tertiary amines display better biocompatibility compared to primary or secondary amines. Nonpolar solvents tend to have better biocompatibility than polar solvents (Kurzrock, 2010). The toxicity of a solvent could also be avoided by applying aqueous two-phase systems (Yang et al., 2007; Planas et al., 1996).

s0150 *Electrodialysis*

- p0255 Electrodialysis is a separation process where ionized compounds are separated from nonionized compounds in aqueous solutions based on transport through ion exchange membranes in an electric field. It has already been used in industry, for example, in the purification of lactic acid from whey, saltwater purification, and glycerine production. An early application on the purification of the carboxylic acid, citric acid, from fermentation broth has been described by Guenther (1963). The conventional monopolar electrodialysis stack consists of an alternating series of anion- and cation-selective membranes separated by flow distribution gaskets, and is often used for concentration and purification through separation of ions and uncharged molecules. The membranes are bound on one end by an anolyte compartment and an anode, while on the other end by a catholyte compartment and a cathode. The direct current moves

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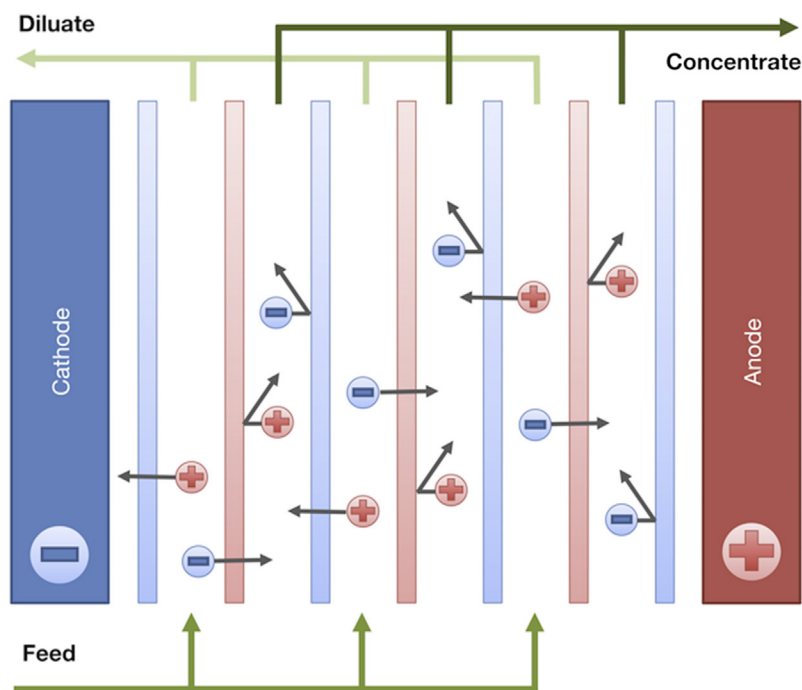


Figure 9.3
Schematic principle of electrodialysis.

the cation through the cation exchange membranes and the anions through the anion exchange membranes. The net result is that salt in the feed compartment is depleted, and the adjacent concentration compartments are enriched with the salt. The negatively charged dicarboxylic acid crosses the anion exchange membrane into the concentrate stream, according to [Fig. 9.3](#). The water splitting bipolar electrodialysis process allows the recovery of the dicarboxylic acid, since it efficiently converts salts into the corresponding alkali and acid, while water is split into H^+ and OH^- . It has the advantage of converting the carboxylate into the corresponding free carboxylic acid without requiring acidifying chemicals, and the recovered base can be reused to control the pH during fermentation. However, the high energy consumption, the cost of the membranes, and the low selectivity for succinic acid are disadvantages. Indeed, the presence of acetate and formate in the fermentation medium hinder the process efficiency due to the inability to selectively separate these acids from succinate ([Song et al., 2007](#)). A more specific problem is the presence of bivalent ions, like calcium and magnesium, often used as neutralization ions during the fermentation, which cause fouling of the electrodialysis membranes. More specifically to water splitting electrodialysis, the low pH of the acid/concentrate stream may cause the crystallization of the dicarboxylic acid on the membrane itself ([Urbanus et al., 2010](#)).

s0155 *Other processes*

p0260 Adsorption techniques include a high number of processes useful for the purification, particularly in polishing steps at the end of DSP of dicarboxylic acids using different solid phase materials, including activated carbon, alumina, silica, and zeolite molecular sieves (Efe et al., 2011; Inci et al., 2012; Jun et al., 2007). Similarly, ion exchange is often applied at the end of entire DSP to remove remaining cations and anions. Simulated moving bed chromatography may be considered to allow for continuous in situ separation during fermentation. The retained dicarboxylic acid can usually be eluted or desorbed with methanol, ammonia, or sulfuric acid (Yang et al., 2007). In general, high-capacity, complete, and low-cost regenerability, as well as specificity, for the target product are desired properties for sorption and ion exchange materials. The pH of the fermentation broth may need to be adjusted before this DSP process, and additional chemicals are needed to regenerate the solid phase (Yang et al., 2007; Joglekar et al., 2006; Thongchul, 2013).

[AU18]

p0265 Further, the distillation of fermentation broths can be used to recover carboxylic acids with a high degree of purity through the separation of volatile and nonvolatile compounds. The distillation processes for the recovery of dicarboxylic acids are based on processing a clarified fermentation broth, free from biomass and insoluble elements, optionally concentrated at high temperatures in distillation apparatuses in order to extract ammonia from the ammonium carboxylates and remove contaminants. The final product is obtained after crystallization by cooling. Operational parameters that influence the process include the pressure, the pH of the medium, the allowed water content in the distillation vessel, the presence of ammonia in the overhead of the distillation and in the liquid phase of the remaining mother liquor, as well as the presence of ammonium carboxylates in the liquid phase. Distillation has the clear disadvantage concerning the energetic costs, and relatively low yields are reported so far. Usually crystallization is used as final purification step, but some cases of direct crystallization are also described in literature (Li et al., 2010; Lin et al., 2010; Luque et al., 2009).

s0160 **9.4.4 Designing an Effective Downstream Process**

p0270 The most important unit operations for the DSP of dicarboxylic acids are discussed in the previous section. Even though they are technically feasible, their industrial realization depends on the ability to scale-up, their robustness, the overall separation yield, investment and operating costs, as well as environmental factors. Further, each process needs to be tailored to the properties of the dicarboxylic acid of interest, to the remaining elements capable of influencing the DSP such as carbon source, or producing microorganisms, and the requirements for yield and purity.

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p0275 In general, recovery and purification of dicarboxylic acids requires a combination of different unit operations. The process should be kept simple with as few steps as possible and be implementable in conventional processing equipment in order to reduce capital and operational expenditure, while at the same time still complying with the required product specification. Importantly, the DSP should not be seen alone but as an integral part of the entire production chain.

s0165 **9.5 Challenges for Sustainable Microbial Production of Dicarboxylic Acids Platform Chemicals**

p0280 A competitive biotechnological production route of dicarboxylic acids needs to consider the following factors:

- u0010 • Low-cost but efficient feedstock: Feedstock can represent a major source of costs for bulk chemicals. Therefore, a cost-effective alternative source for carbohydrates needs to be identified and established in the production process. Waste by-products from eg, chemical sector (such as glycerol from biodiesel production) and waste biomass from eg, agricultural or forestry sectors (such as straw, leftovers after fruit and vegetable processing) could be promising ([Cheng et al., 2012b](#)).
- u0015 • Low organic and inorganic impurities: Several recovery methods are hampered in efficiency in the presence of organic and inorganic impurities, mostly coming from the unclean feedstock. Further, pretreatment of the raw material may need to be implemented, as some of the compounds entrained with the raw material can affect the growth of the producing microorganism.
- u0020 • Optimized fermentation with yeasts: Production of dicarboxylic acids using yeast has several advantages, of which the most relevant is the high yeast tolerance to low pH. This reduces the need for chemical input for pH adjustment and consequently less by-product formation. Moreover, the dicarboxylic acid can predominantly be recovered in the form of free acid if pH is below the pKa (<4.21 for succinic acid).
- u0025 • Increased productivity and yields while at the same time lower production of by-products: A higher concentration of target product results in less extensive DSP. The selective separation of different dicarboxylic acids is difficult if not impossible; thus the reduction of by-product formation during fermentation is relevant. Further, by avoiding the formation of by-products, the carbon source is not significantly diverted toward the production of unwanted products, and thus, the raw material usage is reduced, with both economic and environmental benefits. For this to be possible, a combination of the isolation or design of high-performing strains with the implementation of an optimized fermentation strategy will be required.
- u0030 • Integrated production of other high value-added products: Simultaneous production of additional substances of commercial interest within the same fermentation is a suggested

path toward an efficient biorefinery (Cheng et al., 2012a). Parallel production of propane-1,2-diol and succinate, isoamyl acetate and succinate, or polyhydroxybutyrate and succinate were suggested using *E. coli* (Dittrich et al., 2009; Kang et al., 2010). In the case of industrial production using yeast, another value-added product might be bioethanol that could be produced in the same infrastructure.

- u0035 • Reduced by-product production and biocompatibility of the process: Production of unprofitable by-products during DSP such as calcium sulfate (gypsum) should be avoided. Moreover, the recycling and reuse of effluents needs to be integrated in the process to save costs and reduce the environmental impact of the overall process.
- u0040 • In situ product recovery: The in situ product recovery results in several advantages for the production of dicarboxylic acids. The effect of product inhibition is reduced, along with the requirement for the pH-controlling element (eg, less chemicals). At the same time, productivity may be increased, and the overall number of processing steps may be reduced.

s0170 9.6 Conclusion

p0320 In order to produce biobased dicarboxylic acids capable of competing with the petrochemical process in a cost-effective and sustainable manner, a strategy for an integrated biorefinery system for the entire value chain, including raw material sourcing and treatment, selection or design of an effective microbial strain, design of a robust and scalable fermentation and DSP process, and whenever possible, the integration of these various steps, needs to be considered.

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Abstract

The biotechnological production of biobased dicarboxylic acids has recently become a hot topic in industrial biotechnology, with many investments involved in the development, piloting, and validation at demonstration scale of diverse processes using renewable raw materials. This chapter will review the main markets and applications of commercially relevant dicarboxylic acids and will briefly present their current chemical and biotechnological production processes. The chapter will mainly focus on the particular case of succinic acid. The microbial platforms that have been proposed will be reviewed with emphasis on yeast strains. The basic requirements for setting up and scaling the bioprocess and the required purification strategy to obtain an economically feasible process yielding a product meeting the required specifications will be presented. Throughout the chapter, the specific challenges of using very low-cost raw materials such as agro-industrial residues will be highlighted.

Keywords: 1,4-Butanediol (BDO); Azelaic acid; Biobased chemicals; Citraconic acid; Crotonic acid; Dicarboxylic acids; Diethyl maleate; Downstream processing (DSP); Fermentation; Fumaric acid; Gamma-butyrolactone; Glutaric acid; Glyoxylic acid; Itaconic acid; Maleic acid; Market; Metabolic engineering; Polybutylene succinate (PBS); Renewable raw materials; Succinic acid; Succinic anhydride; Tetrahydrofuran (THF); Yeasts.